MODULATION OF ESTROGEN RECEPTOR FUNCTION BY BRCA1

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Our long-term goal is to understand how mutational inactivation of the breast cancer susceptibility gene, BRCA1, leads to breast tumorigenesis. At the cellular level, BRCA1 ensures global genome stability through its dual participation in DNA double-strand break repair and transcriptional regulation of DNA damage-inducible genes that function in cell cycle checkpoint control. Because the DNA damage-induced signaling pathways that converge on BRCA1 are conserved in most cell types, BRCA1 is likely to function ubiquitously in the maintenance of genome integrity. Nonetheless, germline inactivation of BRCA1 leads principally to cancers of the breast and ovary, and the underlying basis for its tissue-restricted tumor suppressor function thus remains poorly defined. Here, we describe a novel function for BRCA1 in suppressing the ligand-independent transcriptional activity of the estrogen receptor alpha (ERalpha), a principal determinant of the growth and differentiation of breasts and ovaries. In BRCA1-null mouse embryo fibroblasts and BRCA1-deficient human ovarian cancer cells, ERalpha exhibited ligand-independent transcriptional activity that was not observed in BRCA1-proficient cells. Ectopic expression in BRCA1-deficient cells of wild-type, but not clinically validated BRCA1 missense mutants, restored ligand-independent repression of ERalpha in a manner dependent upon histone deacetylase activity. In estrogen-responsive human breast cancer cells, we observed an association between BRCA1 and ERalpha at endogenous estrogen-responsive gene promoters before, but not after, estrogen stimulation. Collectively, these results reveal BRCA1 to be a ligand-reversible barrier to transcriptional activation by unliganded promoter-bound ERalpha, and suggest a possible mechanism by which functional inactivation of BRCA1 could promote tumorigenesis through inappropriate hormonal regulation of breast epithelial cell proliferation. These studies thus offer possible insight into the tissue-specific tumor suppressor function of BRCA1 and suggest defined molecular targets for future intervention in breast cancer.

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CONSTRUCTION AND CHARACTERIZATION OF FIRST- AND SECOND-GENERATION ADENOVIRAL VECTORS EXPRESSING BRCA1 AND MUTANT BRCA1 TRANSGENES

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The protein product of the BRCA1 gene is a ~220 kDa nuclear phosphoprotein. In our experience, detection of ectopically expressed untagged BRCA1 at the protein level is difficult and the documentation provided in many published reports appears unconvincing. The reason for this difficulty is unclear, but may be related to a combination of factors including the large size and/or poor translatability of the BRCA1 mRNA, post-translational control of BRCA1 protein levels, and the relative insensitivity of currently available antibodies. We have circumvented this problem by constructing recombinant adenoviral (Ad) vectors which express human or murine BRCA1 transgenes, as well as a panel of clinically validated human BRCA1 mutants targeting three discrete regions of the BRCA1. Ad vectors expressing BRCA1 mutants C61G (RING finger), 340T (exon 11) and 1853T (BRCT repeats) were constructed. These vectors were capable of transducing a high level of readily detectable BRCA1/mutant BRCA1 protein expression to a variety of human breast and ovarian tumor cell lines in vitro, including hereditary-null breast (HCC1937) and ovarian (SNU-251) tumor cell lines. Normal human mammary epithelial cells (HMEC) and mouse embryo fibroblasts (MEF) were also readily transduced. Functionality of the ectopically expressed BRCA1/mutant BRCA1 protein was examined in several assay systems, including growth-inhibition in vitro and in vivo, DNA damage response, and ubiquitin-ligase associated activities. Overexpression of the BRCA1 protein via an adenoviral vector scored in some of these assay systems, but failed in others. Second-generation Ad vectors expressing the same BRCA1 transgenes but in the context of an Ad vector with a modified Ad fiber knob which facilitates vector particle binding and entry into target cells via a CAR-independent manner were also constructed and evaluated similar to the first-generation vectors. The second-generation Ad vectors were up to 10-fold more efficient in transduction of BRCA/mutant transgene expression to certain target cells when compared at an equivalent viral dose to the first-generation vectors. Results will be discussed in terms of the utility and applicability of Ad-mediated transgene expression in the context of BRCA1 research.

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CHARACTERIZATION OF A TRANSCRIPTIONAL SILENCER AT THE UPSTREAM OF HUMAN BRCA2 GENE PROMOTER

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The expression of the tumor suppressor, BRCA2, is tightly regulated during mammary epithelial cell proliferation and differentiation. BRCA2 is highly expressed during the S and G2/M phases of the cell cycle, but virtually undetectable during the G0 and early G1 phases of the cell cycle. Factors that regulate the temporal and tissue-specific expression of BRCA2 are largely unknown. We have identified a cell cycle-dependant transcriptional silencer at the distalend (-701 to -921) of the human BRCA2 gene promoter. This element is able to silence the activity of cloned human BRCA2 basal promoter while cells are in the G0 and early G1 phases of the cell cycle, however the silencing activity is inhibited when cells enter the S phase. The silencer activity at the G0 phase of the cells varies depending upon the ethnic origin of the human breast cell line, with higher silencer activity in cells from Caucasian origin than those from African-American origin. The mechanism for this variation in silencer activity is currently unknown. Two Alu sequences (-720 to -778 and -860 to -921) and two non-canonical E-box sequences (-706 to -717 and -842 to -853) are the cis-elements present within this silencer, with each Alu sequence preceded by a non-canonical E-box sequence. Single mutants of these sequences displayed silencer activity similar to that of the unmutated element. The double mutants containing defects in both E-box sequence and Alu sequence abbrogated the silencer function. Binding of breast cell nuclear proteins to the silencer was effectively competed out by the E-box sequences but not by mutated E-box sequences, suggesting that those proteins mainly bind to the E-box sequences of the silencer.

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CHARACTERIZATION OF BRCA1-CONTAINING COMPLEXES

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Mutations in the BRCA1 are associated with about 50% of all familial breast cancers. The BRCA1 is known to participate in multiple cellular processes, transcription, DNA repair, cell cycle, and ubiquitination. Our laboratory already established biochemical purification strategy that can partially purify RNA polymerase II holoenzyme (holo-pol) and we have reported that BRCA1 is associated with holo-pol.

In these experiments, we constructed adenovirus vectors to express HA-tagged BRCA1 of full-length and several deletion mutants and infected to large scale cell culture. We prepared whole cell extracts and fractionated using the Biorex70 ion exchange matrix. These fractions were subjected to sucrose gradient sedimentation.

We resolved four distinct BRCA1-containing complexes. We found BRCA1 associated with the holo-pol, a large mass complex called the Fraction 5 complex, the Rad50-Mre11-Nbs1 complex and, new complex, the HUIC (hydroxyurea induced complex) that is a observed after treating cells with hydroxyurea (HU), that causes DNA replication blockage. Following HU treatment of cells, BRCA1 content decreased in the holo-pol and the Fraction 5 complex, and BRCA1 was redistributed to the HUIC. The HUIC was associated with BARD1. These data suggest that BRCA1 participates in multiple cellular processes by multiple protein complexes and that the BRCA1 content of these complexes is dynamically altered following DNA replication blockage.

Furthermore, we characterized BRCA1 association with the holo-pol. BARD1 is a component of the holo-pol. Deletion of the BRCA1 amino-terminus mostly eliminates the association with the holo-pol. Surprisingly, overexpression of the BRCA1 carboxy-terminal deletion mutant resulted in a decrease of hypophosphorylated Pol II. This loss of unphosphorylated Pol II suggests that BRCA1 may regulate the stability of the polymerase. Immunocytochemistry showed that the amino-terminus of BRCA1 is important for nuclear dot formation in S-phase. An intact BRCA1 amino-terminus is required for the association with holo-pol and for subnuclear localization in S-phase foci. An intact BRCA1 carboxy terminus is associated with the stability of Pol II. Taken together, these data support a role for BRCA1 regulation of Pol II function.

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DELAYED MAMMARY TUMOR FORMATION IN CONDITIONAL BRCA1 MUTANT MICE AFTER BILATERAL OOPHORECTOMY

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Germline mutations of the breast cancer suppressor gene 1 (BRCA1) have been found to contribute to about half of the familial breast cancers and most of the combined familial breast and ovarian cancers. The tissue-specificity of the tumorigenesis suggests a role for hormones in BRCA1-associated tumorigenesis. We have recently created a mouse model that carries a conditional mutation of Brca1 in their mammary epithelium through a Cre-LoxP mediated approach. In a p53 heterozygous knockout background, virtually all the mutant female mice (Brca1CO/COMMTV-Crep53+/-) developed mammary tumors between 6-12 months of age. In this study, we investigated the effect of bilateral oophorectomy on mammary tumor incidence in these mice. The mutant females were mated to have 2 full cycles of pregnancy and nursing. After the mating, the animals were about 4 months of age on the average and had approximately 90% of Brca1 transcripts deleted in mammary epithelial cells as indicated by northern blot analysis. We then surgically removed ovaries from 20 mice, and left 39 un-oophorectomized mice as controls. Mice were monitored weekly for the development of palpable tumors and sacrificed within one week of tumor detection. We showed that mammary tumors initated at about same time (200 days) in both groups, and there was a significant decrease (approximately 50% reduction) in the incidence of mammary tumor formatin in the group of oophorectomized mice when they were at arround 330 day of age when compared with the control group. However, the tumor incidence increased significantly in the oophorectomized mice and catched up with that of control group at arround 400 day of age. Because oophorectomy can only delay, but not prevent, mammary tumorigenesis, we conclude that estrogen signals may be involved, but do not play a major role, in mammary tumor formation in Brca1 conditional knockout mice.

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SUSCEPTIBILITY OF BRCA2 HETEROZYGOUS NORMAL MAMMARY EPITHELIAL CELLS TO RADIATION-INDUCED TRANSFORMATION

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Inheritance of one defective copy of the BRCA2 gene predisposes humans to familial, early-onset breast cancer. Recent intensive studies have clearly indicated that BRCA2 plays a very important role in the DNA damage response. In addition to surgery and systemic therapy, radiation therapy is one of the most important components of breast cancer management. Lumpectomy and radiation therapy remains a standard of care option for the majority of patients with early stage breast cancer. It is estimated that about 40-60% breast cancer patients with early stage disease will undergo radiation treatment. However, development of radiation-induced secondary malignancy after successful radiotherapy of a prior cancer has been well documented. It is generally accepted among radiation oncologists that, for most patients, this is an unavoidable complication. However, for specific population of patients such as BRCA2 germline mutation carriers, the risk of radiation induced secondary breast cancer warrants further investigation. Since BRCA2 plays important roles in the DNA damage response, it is reasonable to hypothesize that radiation treatment of breast cancer patients who inherit one defective copy of the BRCA2 gene may pose greater risk of radiation-induced secondary cancer. No study has ever been published to address this important question. The BRCA2 knock out mouse models have very limited value to ratify this hypothesis, since transformation of murine cells is significantly different from that of human cells. Clinical study is hindered by small number of patients with documented BRCA2 status, long term follow up, and interference of multigenic effects. Therefore, in this study, we propose to directly assess the susceptibility of BRCA2 heterozygous normal human mammary epithelial cells to radiation induced transformation. We have constructed a BRCA2 knockout construct, which is designed to disrupt the BRCA2 gene at its exon 11. Starting from a normal human mammary epithelial cell strain, 76N, we are in the process of generating a normal human mammary epithelial cell strain with one allele of BRCA2 disrupted by targeted recombination. The resulting BRCA2 heterozygous cells (BRCA2 +/-) and their parental cells will be an ideal system to examine the susceptibility of BRCA2 +/- cells to radiation induced transformation, since this pair of cell strains will have identical genetic background with the exception of BRCA2 locus. The BRCA2 +/- cells and their parental cells will be treated with fractionated γ-irradiation with a clinically used protocol (50 Gy, 2Gy per working day, a commonly used protocol to treat breast cancer patients). The treated cells will be assessed for their proliferation potential, anchorage independent growth and tumor formation in nude mice.

If data from this study proves that there is no difference between the BRCA2 +/- cells and their parental cells in their susceptibility to radiation-induced transformation, it will strongly suggest that radiation therapy does not pose a higher risk of radiation induced second breast cancer in BRCA2 mutation carrier patients. If data from this study proves that the susceptibility of BRCA2 BRCA2 +/- cells to radiation induced transformation is much higher than their parental cells, it will strongly argue against choosing radiation therapy for BRCA2 carrier patients.

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FUNCTIONAL STUDIES OF BRCA1 AND BRCA2 DURING MITOSIS

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BRCA1 and BRCA2 mutations are associated with familial breast cancer and account for the majority of hereditary breast cancer cases. Therefore, it is important to understand the biological functions of BRCA1 and BRCA2. It is known that BRCA1 is involved in gene transcription, chromatin remodeling, DNA damage repair, and cell cycle control. We have reported a novel finding that BRCA1 is associated with mitotic centrosomes in vivo perhaps through its interaction with gamma-tubulin, suggesting that BRCA1 may play a functional role at mitotic centrosomes. Here we have in vitro evidence indicating that indeed full-length BRCA1 protein generated by in vitro transcription/translation interacts with gamma-tubulin. We have also identified a region of BRCA1 protein, BF3 (amino acids 504-803), which is necessary and sufficient for binding to gamma-tubulin. BF3 and gamma-tubulin coimmunoprecipitate when expressed in COS-7 cells. Immunoprecipitation experiments also demonstrate that overexpression of BF3 interferes with the association of full-length BRCA1 protein and gamma-tubulin, and therefore may have a dominant negative effect on BRCA1 function(s). Overexpression of BF3 in COS-7 cells results in growth suppression and induction of apoptosis, which may be p21-dependent. In addition, overexpression of BF3 also induces an accumulation of mitotic cells with multiple centrosomes and abnormal spindles. In summary, we have mapped a gamma-tubulin-binding domain in BRCA1 and overexpression of this domain results in accumulation of mitotic cells with multiple centrosomes which eventually may undergo apoptosis or tumorigenesis.

Furthermore, we have also demonstrated that BRCA2 interacts with gamma-tubulin and is associated with the centrosome during mitosis. Therefore, BRCA2 may have a similar function as BRCA1.

In conclusion, BRCA1 and BRCA2 may play a functional role at mitotic centrosomes through the regulation of centrosome duplication/centrosome integrity, mitotic spindle formation and proper segregation of chromosomes during mitosis, and help maintain the fidelity of cell division and preserve genomic stability.

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REGULATION OF BRCA1 DEPENDS ON P53 STATUS FOLLOWING TREATMENT WITH CISPLATIN

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Germline mutation in *BRCA1* is responsible for approximately half of all cases of hereditary breast cancer and for almost all combined hereditary breast and ovarian cancers cases. Recent studies suggest that BRCA1 plays an important role in maintenance of genomic stability through DNA repair. Cisplatin is a DNA damaging reagent that crosslinks with DNA. Because different DNA-damaging agents can be involved in different DNA repair pathways, we hypothesize that the DNA-crosslinking reagent cisplatin may involve a different DNA repair pathway than that of ionizing irradiation. To test this hypothesis, we used three cancer cell lines: SNU251 containing both p53 and BRCA1 mutations, SKOV3 carrying only a p53 mutation but containing wild-type BRCA1, and OVCA433 bearing both wild-type p53 and wild-type BRCA1. All three cell lines were treated with cisplatin or γ-radiation. Our results demonstrated that BRCA1 expression levels were upregulated in the p53 mutation cell lines SKOV3 and SNU251 but is downregulated in the wild-type p53 cell line OVCA433 following cisplatin treatment. On the other hand, only minimal changes in BRCA1 expression levels were detected in all three cell lines following γ-radiation. These results provided experimental evidence that cisplatin regulated the expression of BRCA1. Such regulation may depend on the status of p53 mutation. Our data also showed that after cisplatin treatment, wild-type BRCA1 was phosphorylated in SKOV3 cells, which are cisplatin resistant. Further study is in progress to reveal the underlying mechanism of the role of BRCA1 in cisplatin-resistant cancer cells.

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BRCA2 EXPRESSION IN SPORADIC INVASIVE MAMMARY CARCINOMA

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Women with germline mutations in BRCA2 have a high likelihood of developing breast cancer. However, mutations in the BRCA2 gene are extremely infrequent in sporadic breast despite the fact that loss of heterozygosity for the BRCA2 gene has been reported in 20-67% of sporadic breast cancers. This suggests that while BRCA2 may not be mutated in these cases, it still may play an important role in the development of sporadic breast cancer. To address this question we have attempted to assess the level of BRCA2 protein and mRNA in a collected series of sporadic breast cancer cases. Initially, we focused on developing antibody reagents to semiquantitatively assay BRCA2 protein by immunohistochemistry. Thus far, we have been unable to develop antibodies that reliably detect BRCA2 in formalin-fixed, paraffin-embedded tissues. Primarily, these attempts have been hampered by unacceptably low sensitivity and specificity of the antibody reagents. Therefore we have concentrated our efforts on measuring BRCA2 mRNA via cDNA microarray. This technology has a number of advantages including the ability to simultaneously assess the expression level of thousands of genes and to utilize relatively small samples. To take full advantage of this capability we have paired microarray technology with laser capture microdissection of tumor samples to insure that we can specifically measure BRCA2 mRNA levels in tumor cells and avoid contamination by adjacent normal breast tissue and tumor stroma. Our results suggest that their is a subset of sporadic breast tumors that exhibit low levels of BRCA2 expression and we hope to correlate expression levels with response to radiation therapy in follow up studies. Studies from cell lines derived from BRCA2 hereditary cancer cases have demonstrated increased sensitivity to radiation and we expect that low BRCA2 expression may identify breast tumors with enhanced radiosensitivity.

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EXTRACELLULAR MATRIX SUPPRESSES BRCA1 EXPRESSION THROUGH ITS C-TERMINAL AND ENHANCES HEREGULIN-DEPENDENT BRCA1 PHOSPHORYLATION

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Germline mutations in the breast cancer susceptibility gene BRCA1 account for the increased risk of early onset of familial breast cancer, whereas overexpression of the ErbB family of receptor tyrosine kinases has been linked to the development of non-familial or sporadic breast cancer. BRCA1 encodes a nuclear phosphoprotein that acts as a tumor suppressor, while activation of the ErbB receptors by heregulin (HRG) promotes cell growth in breast epithelial cells. We aimed to determine the effects of ErbB2 activation on BRCA1 function. Our studies showed that HRG induced the phosphorylation of BRCA1, which was mediated by the phosphatidylinositol-3 kinase (PI3K)/Akt pathway. Since altered interaction between cells and surrounding extracellular matrix (ECM) is a common feature in a variety of tumors and ECM modulates intracellular signaling, we hypothesize that ECM may affect the expression and HRG-dependent phosphorylation of BRCA1. Following stimulation by HRG (20 nM), a strong increase in [3H]thymidine incorporation, peaking between 12 and 18 h, was observed in human T47D breast cancer cells seeded on plastic (PL). When T47D cells were seeded on laminin (LM) or matrigel, HRG induced a significantly higher mitogenic response comared to cells seeded on plastic. Cells seeded on collagen IV (CL4) had very modest response to mitogenic stimuli. Furthermore, T47D cells seeded on poly-L-lysine (POL), had abrogated mitogenic response, indicating the involvement of integrins in this process. In addition, HRG treatment induced a transient phosphorylation of BRCA1, peaking between 0.5-1 h, that was enhanced in T47D cells grown on LM, while the phosphorylation of BRCA1 was much lower in cells seeded on POL or in cells maintained in suspension. Accordingly, cells grown on LM had the greatest increase in ErbB2 activation, PI3K activity, and phosphorylation of AKT. A similar pattern of mRNA expression of BRCA1 was observed when T47D cells were seeded on PL, LM and CL4. Interestingly, there was a significant decrease in the steady state of the BRCA1 mRNA level on both matrices LM and CL4 as compared to cells seeded on PL. HCC-1937 breast cancer cells, that contain a truncated C-terminal and therefore non-functional BRCA1 protein, did not affect BRCA1 RNA expression.

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RESTORING BRCA1 FUNCTION WITH ANTIBIOTICS

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Approximately 45% of all families with hereditary breast and ovarian cancer have germ-line mutations in BRCA1. These are highly penetrant mutations and confer a 56-85% lifetime risk of developing cancer. Linkage analysis suggest that all mutations leading to premature termination of the protein will confer high cancer risk. Some individuals carry germ-line nonsense mutations and are therefore highly susceptible to cancer. Recent studies have shown that aminoglycosides suppress nonsense mutations in mammalian transcripts and partially restore the functional protein. Therefore, individuals carrying germ-line nonsense mutations in BRCA1 could benefit from the ability of aminoglycosides to induce translation readthrough and restore the function of BRCA1.

We investigated whether aminoglycosides would induce translation readthrough in BRCA1 nonsense mutations, exploiting the ability of the C-terminal region of wild-type BRCA1 to activate transcription when fused to heterologous DNA-binding domains (DBD). A nonsense mutation (Y1769X) was introduced by site-directed mutagenesis into a segment of BRCA1 containing exons 13-24 fused to GAL4 DBD. The expression vector containing the mutant and constructs carrying negative and positive controls were transfected into a human embryonic kidney cell line (293T) with a GFP reporter under the control of GAL4 binding sites. Transfected cells were then treated with several aminoglycosides and expression of GFP was assessed.

As expected, wild-type BRCA1 induced a high expression of GFP in untreated and treated cells. Conversely, a construct carrying a missense cancer-associated mutation (A1708E) was unable to induce detectable GFP expression either in treated or untreated cells. Interestingly, while constructs carrying the nonsense mutation did not induce detectable expression in untreated cells, treatment with aminoglycosides partially restored BRCA1 function, as measured by GFP expression.

Conclusion: Aminoglycosides were able to partially restore transcription activation by a nonsense mutant of BRCA1 in human cells. These results corroborate previous findings that aminoglycosides suppress nonsense mutations and suggest that it could be used as an option of preventive regimen to individuals with nonsense mutations in BRCA1.

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MUTATIONS IN THE HYDROPHOBIC CORE OF THE BRCT DOMAIN CONFER TEMPERATURE SENSITIVITY TO BRCA1 IN TRANSCRIPTION ACTIVATION

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The breast and ovarian cancer susceptibility gene BRCA1 is a tumor suppressor and germ line mutations in this gene account for the majority of familial cases of breast and ovarian cancer. The precise biochemical function of BRCA1 is still unknown but there is mounting evidence indicating its involvement in two fundamental cellular processes: DNA repair and transcriptional regulation. A major hurdle to dissect the role of BRCA1 is the lack of molecular biological tools to carry out biochemical and genetic experiments.

To fill this gap we undertook a differential screen in yeast to isolate temperature-sensitive (TS) mutants of BRCA1 in transcription activation. Using a random mutagenesis approach we generated mutants of the carboxy-terminal region of BRCA1 (aa 1560-1863) that display a temperature-dependent activity in yeast transcription assays. We obtained 8 unique mutants that display wild-type activity in transcription at 30ºC but markedly reduced activity at 37ºC. In addition we were also able to identify 11 unique missense mutations that resulted in a loss-of-function phenotype at both temperatures. Mutations resulting in temperature-dependent activity were primarily located in the hydrophobic core of the BRCT domain of BRCA1. All TS mutants were subsequently analyzed in mammalian cells and showed loss of function at both temperatures suggesting that both the range of temperature and promoter stringency have to be adapted in order to generate TS mutants for mammalian cells. These conditional mutants will represent important tools to assess the role of BRCA1 in transcription in yeast and may form the basis to develop similar tools for mammalian cells.

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THE EFFECT OF HETEROZYGOSITY FOR A MUTATION IN BRCA1 ON MAMMARY TUMOR DEVELOPMENT IN *APC*^{MIN}/+ MICE FOLLOWING IRRADIATION

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BRCA1 was identified due to an increased risk of breast and/or ovarian cancer in females who carry a mutant allele. It has been proposed that BRCA1 has a role in DNA damage repair. If this is the case, women with mutations in *BRCA1*, who have only one functional allele, may have increased susceptibility to the effects of DNA damaging agents, such as ionizing radiation. This susceptibility may result in an increased mutation rate after exposure to irradiation. Planning of prevention and treatment strategies for such high risk women must include information about environmental exposures that might increase risk. Mouse models can provide a good system in which to investigate a question such as this. We are testing for the effect of a *Brca1* mutation on mammary tumor development in *Apc*^{Min}/+ mice, which have an increased susceptibility to mammary tumor development. The number of mammary tumors in *Apc*^{Min}/+ mice is increased by exposure to carcinogens or irradiation. The hypothesis that we are testing is that if heterozygosity for a mutation in *Brca1* increases the sensitivity to the effects of irradiation, we expect that the double mutant mice would develop more tumors or more advanced tumors after irradiation than the mice carrying either mutant allele alone. We have generated mice carrying mutant alleles at both *Brca1* and *Apc* and determined the number and time to tumor after irradiation. We have tested multiple doses of irradiation and also the effect of multiple exposures. The results of these experiments will be presented.

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REGULATION OF BRCA1 BY PHOSPHORYLATION

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Germline mutations of the BRCA1 gene are responsible for many cases of hereditary breast cancer. The BRCA1 protein is a 220-kDa nuclear phosphoprotein protein, which is expressed and phosphorylated in late G1, S and G2 phases. In response to DNA damage BRCA1 is hyperphosphorylated and changes sub-cellular location. Since BRCA1 is phosphorylated in a cell cycle and DNA damage dependent manner, phosphorylation may play a regulatory role for the function of the BRCA1 protein.

Understanding the role of phosphorylation of BRCA1 will be the next logical step for a complete understanding of BRCA1 functions. The identification of in vivo phosphorylation sites is therefore the prerequisite for this effort. It has been technically changeling with traditional techniques to identify in vivo phosphorylation sites for the BRCA1 protein, as it is not an abundant protein. However, recent advance in mass spectrometry has made this task within reach. I proposed in this grant to identify in-vivo phosphorylation sites of the endogenous BRCA1 protein without exogenous DNA damage from asynchronized cells and study their functional significance by site-directed mutagenesis and complementation of the breast cancer cell line HCC1937, in which the BRCA1 protein is truncated and its expression is much lower than normal cells.

We made great effort to isolate sufficient amount of the BRCA1 protein from cycling HeLa cells so that we can analyze phosphorylation of the protein and identify the exact phosphorylation sites by mass spectrometry. BRCA1 is so scarce that we can only purify ~ 200 ng of the protein from ~ 50 L Hela cells. This amount of the protein (~ 1 pmol) allowed us to identify a single phopshorylation site by mass spectrometry each time that we purified sufficient amount of the proteins. We managed to identify one in vivo phosphorylation site as S1189. S1189 does not confer to phosphorylation concensus of any known BRCA1 kinases, suggesting that BRCA1 is subject to regulation by a novel BRCA1 kinase. Mutagenesis of this site and complimentation of the HCC1937 cell lines are underway to assess the functional significance of this phosphorylation site.

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IDENTIFICATION OF BRCA2-ASSOCIATED PROTEINS

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Mutations in the tumor suppressor gene BRCA2 are responsible for the same percentage of familial breast cancers as BRCA1.The mechanisms by which BRCA2 suppresses tumor formation in normal tissues are not understood and the sequence of the BRCA2 gene provides few clues. We proposed to purify and define the BRCA2 protein complex using antibody affinity chromatography and mass spectrometry in an effort to understand the function of the BRCA2 protein.

In the past two years, we generated and selected several antibodies that can immunoprecipitate the BRCA2 protein, which allowed us to use the proposed method in the grant to identify BRCA2 associated protein. We immunoprecipitated the BRCA2 protein from HeLa nuclear extracts, resolved the immuno-precipitates on SDS-PAGE and identified the co-precipitated proteins by mass spectrometry. It appears that the BRCA2 complex is heterogeneous in the cell, as different BRCA2 antibodies can precipitate different associated proteins. We have identified several proteins from one polyclonal antibody BRCA2-2B, in which at least two proteins, BRAF35 and BubR1 were reported to interact with the BRCA2 protein, indicating that these proteins may be specific BRCA2 associated proteins. Most of them seem to function in mitosis, which may suggest that BRCA2 functions in the mitotic checkpoint to play the caretaker role to maintain the genome. Most surprisingly, we also identified the RAD50/MRE11/NBS1 protein complex (the M/R/N complex) that co-precipitates with the BRCA2 protein from an antibody that was raised against the C-terminal peptide of the BRCA2 protein. We have confirmed that this association is specific, as multiple BRCA2 antibodies can precipitate the M/R/N complex. It is known that the M/R/N complex interacts with BRCA1, and BRCA1 interact with BRCA2, so it is possible that association of the M/R/N complex with BRCA2 depends on BRCA1. However, further experiments suggest that this is not the case, as the BRCA2 antibody precipitates more M/R/N complex than the BRCA1 antibody. It is clear that many more experiments are needed to comprehend the functional significance of these BRCA2 associated proteins.

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THE ACTIVATED AROMATIC HYDROCARBON RECEPTOR REPRESSES BRCA-1 PROMOTER ACTIVITY AT XENOBIOTIC RESPONSIVE ELEMENTS

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The purpose of this project is to investigate whether or not loss of expression of the BRCA-1 gene in breast epithelial cells exposed to polycyclic aromatic hydrocarbons (PAHs) is mediated by the aryl hydrocarbon receptor (AhR). The hypothesis being tested is that the AhR complexed with the AhR-nuclear transporter (ARNT) protein, binds to several xenobiotic responsive elements (XRE) strategically located at –539 bp (CGTGGAA=CYP1A1-like) and +20base pairs (bp) (CGTG=XRE-1) from the transcription start site on exon-1A. Two additional XREs (CGTG) have been localized at –107 bp in the intervening sequence upstream (XRE-2) and +218 bp (XRE-3) into exon-1B. In transfection experiments, we observed that treatment of estrogen receptor positive MCF-7 cells with benzo[a]pyrene (B[a]P) repressed transcription at the BRCA-1 promoter. To clarify the contribution of the various candidate XRE-binding domains to repression of BRCA-1 transcription, we developed single, double and triple mutation- and deletion-constructs for the CYP1A1-like and XREs. We report that deletion of the CYP1A1-like and XRE-1 core sequence reduced (1.6- and 2.0-fold) basal, whereas deletion of XRE-2 increased (3.0-fold) both basal and estrogen-induced, transcription activity of the BRCA-1 promoter. Transfection experiments with mutation constructs (CGTG to CcaG or Ccaa) indicated that XRE-1 may function as a negative cis-acting element of basal BRCA-1 transcription. In electromobility shift experiments with nuclear extracts obtained from MCF-7 cells treated with B[a]P, we observed that the AhR contributed to the formation of a transcription complex at the XRE-1 site. These cumulative data suggested that the activated AhR may contribute to disrupting basal and estrogen-regulated BRCA-1 transcription. The significance of these findings is that exposure to environmental pollution containing PAHs and ligands of the AhR may be a risk factor in the etiology of sporadic breast cancer by disrupting BRCA-1 expression, thus compromising the DNA repair capacity of cells.

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DISRUPTION OF BRCA1 LXCXE MOTIF ALTERS BRCA1 FUNCTIONAL ACTIVITY AND REGULATION OF RB FAMILY, BUT NOT RB, PROTEIN BINDING

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The tumor suppressor activity of the BRCA1 gene product is due, in part, to functional interactions with other tumor suppressors, including p53 and the retinoblastoma (RB) protein. RB binding sites on BRCA1 were identified in the C-terminal BRCT domain (Yarden and Brody, 1999) and in the N-terminus (aa 304-394) (Aprelikova et al., 1999). The N-terminal site contains a consensus RB binding motif, LXCXE (aa 358-362), but the role of this motif in RB binding and BRCA1 functional activity is unclear. In both in vitro and in vivo assays, we found that the BRCA1:RB interaction does not require the BRCA1 LXCXE motif, nor does it require an intact A/B binding pocket of RB. In addition, nuclear co-localization of the endogenous BRCA1 and RB proteins was observed. Over-expression of wild-type BRCA1 (wtBRCA1) did not cause cell cycle arrest but did cause down-regulation of expression of RB, p107, p130, and other proteins (e.g., p300), associated with increased sensitivity to DNA-damaging agents. In contrast, expression of a full-length BRCA1 with an LXCXE inactivating mutation (LXCXE-->RXRXH) failed to down-regulate RB, blocked the down-regulation of RB by wtBRCA1, induced chemoresistance, and abrogated the ability of BRCA1 to mediate tumor growth suppression of DU-145 prostate cancer cells. wtBRCA1-induced chemosensitivity was partially reversed by expression of either Rb or p300 and fully reversed by co-expression of Rb plus p300. Our findings suggest that: (1) disruption of the LXCXE motif within the N-terminal RB binding region alters the biologic function of BRCA1; and (2) over-expression of BRCA1 inhibits the expression of RB and RB family (p107 and p130) proteins.

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THE ROLE OF BREAST CANCER SUSCEPTIBILITY GENE 1-DEPENDENT UBIQUITINATION IN BREAST CANCER

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Mutational inactivation of the Breast Cancer Susceptibility Gene, *BRCA1*, accounts for a large percentage of hereditary breast cancer. BRCA1 has been shown to function in a number of different cellular processes, yet it is still unclear how BRCA1 biochemically mediates its cellular function as a tumor suppressor protein. Recently, the highly conserved ring finger domain of BRCA1 has been implicated in the ubiquitination of proteins and is thought to function as a ring finger-type ubiquitin protein ligase or E3 enzyme. However, how the putative E3 activity of BRCA1 may mediate the functions of BRCA1 in the cell remains unclear. Our hypothesis is that BRCA1 mediates its biological function by targeting proteins for ubiquitination. We propose that BRCA1 effects the ubiquitination of proteins that are either negative regulators of DNA repair or are positive regulators of growth proliferation, leading either to their degradation or to an alteration of their activity.

In an attempt to understand the biological significance of BRCA1-dependent ubiquitination, we have generated the reagents to reconstitute BRCA1-dependent ubiquitination using E1 (Uba1), E2 (UbcH5b), and full-length recombinant BRCA1 expressed in baculovirus. We are also currently expressing full-length BARD1 in baculovirus. To determine whether BRCA1 and BARD1 truly function as an ubiquitin protein ligase, we will determine whether the full-length BRCA1 and BARD1 heterodimer functions to ubiquitinate bacterial proteins or exhibits auto-ubiquitination activity. It is expected that only full-length BARD1 and BRCA1 proteins will confer physiologically relevant substrate specificity, and thus, these full-length proteins will be used to identify substrates of BRCA1 using an *in vitro* ubiquitination assay. We are currently testing Estrogen Receptor α, RNA Polymerase II large subunit, and CDC25A as potential BRCA1/BARD1 substrates. In the event that E1, E2, and BRCA1/BARD1 are not sufficient for the ubiquitination of putative substrates *in vitro*, we are also generating nuclear extracts from cell lines deficient in the expression of BRCA1 to provide proteins that may be additionally required for BRCA1-dependent ubiquitination.

This study addresses a highly novel putative activity of BRCA1 that could have a large impact on our understanding of familial breast cancer development. It is our hope that these studies will identify a previously uncharacterized biochemical activity of BRCA1 in the cell and several important targets of this BRCA1 activity. This discovery could not only alter the manner in which all future studies are directed on the function of BRCA1 in the cell, but could also open many new avenues for the development and implementation of drugs for the treatment of breast cancer.

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TRANSCRIPTIONAL ACTIVATION OF GADD45 BY BRCA1

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Breast cancer susceptibility gene BRCA1 has been implicated in the control of gene regulation and such regulated genes are thought to mediate the biological role of BRCA1. Overexpression of BRCA1 induces GADD45, a p53-regulated and stress-inducible gene. However, the molecular mechanism(s) by which BRCA1 regulates GADD45 remains to be elucidated. In this report, we have shown that the GADD45 promoter is strongly activated following expression of wild-type BRCA1. In contrast, both the tumor-derived BRCA1 mutants (p1749R and Y1853insA) and truncated BRCA1 mutant protein (D500-1863 BRCA1), which lack transactivation activity, were unable to activate the GADD45 promoter, indicating that the BRCA1-mediated activation of the GADD45 promoter requires normal transcriptional properties of BRCA1. BRCA1 did not induce the c-Jun and c-fos promoters, which rules out a general effect of BRCA1 on other immediate-responsive genes. Expression of the HPVE6 and the dominant-negative mutant p53 proteins had no effect on the induction of the GADD45 promoter by BRCA1, suggesting that activation of the GADD45 promoter by BRCA1 is independent of cellular p53 function. With the 5'-deletion analysis, the BRCA1-responsive element of the GADD45 promoter was mapped at the region from -107 to -57. Disruption of this region resulted in abrogation of BRCA1 activation of the GADD45 promoter. Sequence analysis exhibits two Oct-1 motifs and one CAAT site in this region and site-directed mutations of both OCT-1 and CAAT motifs abolished induction of the GADD45 promoter by BRCA1. Importantly, physical associations of BRCA1 protein with transcription factors Oct-1 and NF-YA, which directly bind to the OCT-1 and CAAT motifs, were established by biotin-streptavidin pull-down and coimmunoprecipitation assays. Such protein interactions were required for interaction of BRCA1 with the GADD45 promoter since either immunodepletion of Oct-1 and NF-YA proteins or mutations in the OCT1and CAAT motifs disrupt BRCA1 binding to the GADD45 promoter. These results demonstrate that GADD45 acts as one of the BRCA1-regulated genes, and indicate that BRCA1 can upregulate its targeted genes through protein-protein interactions and provide a novel mechanism by which BRCA1 participates in transcriptional regulation.

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